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13. ABSTRACT (Maximum 200 words) Interaction of the estrogen receptor with its ligands is mediated by a C-terminal region designated the hormone binding domain (HBD). We initiated structure-function studies in an attempt to improve our understanding of how estrogen activates the receptor and how antagonists inhibit its activity. We previously reported high yield expression of recombinant human estrogen receptor HBD and suggested that the isolated HBD forms dimers in solution and undergoes conformational changes comparable to those in the full-length protein. Small crystals of the HBD have been obtained, although these are not yet suitable for diffraction analysis. Structural modeling of the HBD predicts that the core of the HBD remains essentially unaffected by ligand binding. Fluorescence spectroscopy results also suggest that the HBD core is largely unaffected by ligand binding: all three of the HBD tryptophan residues appear to be located in hydrophobic environments in the presence and absence of ligand. Studies using iodide as a quenching agent showed that estradiol binding increased the accessibility of Trp360, suggesting that ligand binding alters the conformational flexibility of the HBD. The modeling and fluorescence studies suggest that the major conformational changes induced by ligand binding may be confined to the N- and C-termini.				
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FOREWORD

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Lang E Vickery 9-29-99

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INTRODUCTION

The research conducted was aimed at improving our understanding of basic molecular mechanisms underlying the biochemical actions of estrogens and at providing guidelines for rational drug design for treatment of estrogen-dependent diseases such as breast cancer.

Characterization of the mechanism of estrogen binding to the receptor will provide new, fundamental information about steroid-protein interactions. Identification of the types of amino acid residues involved, the contacts and bonds formed, and whether there exists a specific protein fold to form a "steroid pocket" will further our understanding of general principles of molecular recognition. Structural information on the HBD should also help us understand how it operates as a ligand-regulated switch to modulate gene transcription. Little is known about how ligand binding affects the conformation of steroid hormone receptors or how this is linked to effects on other domains of the receptor. Comparison of the structure of the HBD bound to agonists versus bound to antagonists should provide insight into the regulatory mechanisms involved.

These studies will also have immediate relevance to the development of drugs for the treatment of estrogen-dependent cancers. Breast cancer is an especially important target because of its high incidence and the substantial response seen with anti-estrogen therapies in a significant percentage of cases. Knowledge about how agonists and antagonists are recognized by the receptor and how their binding results in differential activation of the receptor should greatly aid in the rational design of ligands which are specific for the estrogen receptor, which bind with high affinity, and which maximize the antagonistic properties. It may also suggest possible structural modifications of known drugs, e.g. tamoxifen, which will favor their metabolism to antagonistic forms over their conversion to estrogenic forms. In addition, the findings will have general relevance to other steroid hormone receptors (e.g. androgen receptor, progesterone receptor) which are also important in a number of cancers.

The 1998 Progress Report, prepared at the end of the regular 4-year award period, provided a detailed account of our findings. In this final report we have summarized the accomplishments associated with each specific Task as outlined in the approved Statement of Work.

BODY

Task 1. Overproduction HBD peptides (Years 1-2). Different expression vectors will be tested to optimize the yield and purification of the recombinant protein in *E. coli*. We will also vary the positions of the first and last residues of the peptides to better define the boundaries of the HBD and to generate the smallest, active truncated fragment.

This task was completed, and was reported in previous progress reports.

Protein expression and purification: The maltose binding protein expression vector primarily used in these studies resulted in high level overexpression of soluble estrogen receptor hormone binding domain. Other vectors tested either did not express the protein in high levels, or expressed the protein in insoluble form.

The purification method finally used (described in previous progress reports, and in Ref. 1) allowed routine purification of wild-type HBD peptide in 40-50% yield. The peptide produced using this method exhibited essentially quantitative estradiol binding, suggesting that the peptide was purified to near homogeneity, and that the peptide preparations consisted of properly folded protein.

Truncation experiments: We constructed a variety of truncation mutants. The results of these studies (reported in previous progress reports) indicated that the amino acid 300-551 construct used for the majority of the studies was not appreciably larger than the minimum active fragment.

N-terminal truncations that involved removing more than six residues were unstable. In addition, peptides that began with positions between 301 and 305, while stable, were more difficult to cleave from the fusion protein and exhibited somewhat altered behavior in dimer interaction studies and in studies of cooperative ligand binding. Thus, it appears that, while the construct beginning at position 300 is not the absolute minimum construct, few, if any additional residues can be removed without altering the structure of the protein.

C-terminal truncations experiments also showed that constructs ending at positions 529 and 534 were unstable. Although examination of the crystal structure had suggested that these truncations should allow removal of a terminal helix that was not a permanent part of the core of the protein. Based on these results, we conclude this C-terminal helix has a role in stabilizing the structure.

The overall conclusion from these experiments was that only at most 20 residues might be removed from the HBD peptide, and these only at the cost of generating peptides with either reduced stability or at least slightly perturbed structure. The

300-551 construct therefore appears to encompass the actual hormone domain with little in the way of additional sequences at either end.

Task 2. Characterization of HBD peptides (Years 1-4). The purified proteins will be characterized with respect to their affinity and stoichiometry for estradiol binding. The relative affinities for other agonists and antiestrogens will also be determined. The spectral properties (absorption, circular dichroism, fluorescence spectra) of the different HBD proteins will be measured, and effects of ligand binding on these properties will be investigated. The polymerization state of the purified HBD, e.g. homodimer or oligomer, and effects induced by ligand binding will also be determined.

These studies were completed and reported in previous progress reports and Ref. 1.

Briefly, the expressed HBD peptide exhibited estrogen binding and ligand discrimination properties similar to those of the full-length protein. The HBD was shown to form dimers in solution, with an affinity inferred to be ~1 nM. Ligand binding significantly reduced the dissociation rate constant.

Spectroscopic studies indicated that absorbance and circular dichroism spectra were unaffected by the binding of estradiol or tamoxifen. Fluorescence spectra indicated that binding of estradiol had minor effects on the environment of one of the three intrinsic tryptophan residues, while tamoxifen and 4-hydroxytamoxifen binding resulted in quenching of all of the HBD tryptophans. The results of the fluorescence studies on wild-type HBD and on tryptophan to phenylalanine mutant forms of the HBD were reported in previous progress reports.

Task 3. Define the roles of specific amino acids (Years 2-5). The contribution of specific residues to any spectroscopic changes induced by ligand binding to the HBD protein will be investigated using site-directed mutagenesis. If the HBD protein behaves as a dimer (or other oligomer) in solution, site-directed mutagenesis will also be used to identify regions mediating the interaction.

Site-directed mutagenesis of residues predicted to be involved in dimer formation was performed. However, most of these mutants resulted in protein that appeared to be either inactive in crude cell lysates, or which was insufficiently stable to allow purification. These experiments did not appear likely to yield promising results based on structural information available during the period of the grant, and were not pursued further.

Task 4. Crystallize the HBD (Years 2-5).

Efforts will be made to obtain crystals of both ligand-free and ligand-complexed forms of the receptor for X-ray-diffraction analysis. Various crystallization conditions will be screened, and HBD proteins varying in size will be tested.

Extensive efforts aimed toward crystallization of the wild-type HBD and wild-type MBP-HBD fusion protein were unsuccessful. Crystallization attempts for a number mutant forms of the HBD were also unsuccessful. As reported in the most recent progress report, the 4-hydroxytamoxifen-bound forms of a cysteine mutant (C381S) did form crystals. However, diffraction quality crystals of this construct were not obtained prior to the ending of the grant.

KEY RESEARCH ACCOMPLISHMENTS

- ◆ High level expression of the hormone binding domain (HBD) of the human estrogen receptor
- ◆ Characterization of the minimal length functional fragment of the HBD
- ◆ Established that dimerization functions of the human ER reside are specified by the HBD region
- ◆ Established that the isolated HBD undergoes conformational changes required for ligand binding cooperativity
- ◆ Dimer formation does not require bound ligand, but both agonists (estradiol) and antagonists (4-hydroxytamoxifen) alter receptor dimerization kinetics

REPORTABLE OUTCOMES

Manuscripts

Brandt, M.E. and Vickery, L.E. (1997) "Cooperativity and dimerization of recombinant human estrogen receptor hormone-binding domain", *J. Biol. Chem.* **272**, 4843-4849.

Brandt, M.E. and Vickery, L.E. (1999) "Structural Effects of Estrogen Receptor Ligand Binding Probed by Tryptophan Fluorescence" in preparation.

Abstracts

Brandt, M.E. and Vickery, L.E. Estrogen Receptor Hormone Binding Domain Cooperativity and Dimerization. Breast Cancer Research Meeting: An Era of Hope, Washington, D.C..

Employment

Co-Investigator Mark E. Brandt, Ph.D., accepted a position as an Instructor in the Department of Chemistry and Biochemistry at the California State University, Fullerton.

CONCLUSIONS

The isolated, recombinant hormone binding domain (amino acid residues 300-551) of the human estrogen receptor exhibits ligand binding, discrimination, and cooperativity functional properties similar to the natural full-length receptor protein. It should provide a useful system for development and characterization of anti-estrogen compounds and drugs to be used to treat estrogen-dependent breast cancer.

REFERENCES

1. Brandt, M.E. and Vickery, L.E. (1997) "Cooperativity and dimerization of recombinant human estrogen receptor hormone-binding domain", *J. Biol. Chem.* **272**, 4843-4849.

APPENDICES

Publication Reprint

Brandt, M.E. and Vickery, L.E. (1997) "Cooperativity and dimerization of recombinant human estrogen receptor hormone-binding domain", *J. Biol. Chem.* **272**, 4843-4849.

Abstract Reprint

Brandt, M.E. and Vickery, L.E. Estrogen Receptor Hormone Binding Domain Cooperativity and Dimerization. Breast Cancer Research Meeting: An Era of Hope, Washington, D.C. (in press).

Cooperativity and Dimerization of Recombinant Human Estrogen Receptor Hormone-binding Domain*

(Received for publication, September 27, 1996, and in revised form, November 26, 1996)

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The estrogen receptor dimerizes and exhibits cooperative ligand binding as part of its normal functioning. Interaction of the estrogen receptor with its ligands is mediated by a C-terminal hormone-binding domain (HBD), and residues within the HBD are thought to contribute to dimerization. To examine dimer interactions in the isolated HBD, a human estrogen receptor HBD fragment was expressed in high yield as a cleavable fusion protein in *Escherichia coli*. The isolated HBD peptide exhibited affinity for estradiol, ligand discrimination, and cooperative estradiol binding (Hill coefficient ~1.6) similar to the full-length protein. Circular dichroism spectroscopy suggests that the HBD contains significant amounts of α -helix (~60%) and some β -strand (~7%) and that ligand binding induces little change in secondary structure. HBD dimer dissociation, measured using size exclusion chromatography, exhibited a half-life of ~1.2 h, which ligand binding increased ~3-fold (estradiol) to ~4-fold (4-hydroxytamoxifen). These results suggest that the isolated estrogen receptor HBD dimerizes and undergoes conformational changes associated with cooperative ligand binding in a manner comparable to the full-length protein, and that one effect of ligand binding is to alter the receptor dimer dissociation kinetics.

The estrogen receptor is a member of a superfamily of nuclear proteins that includes the receptors for the steroid hormones, for vitamins A and D, and for thyroid hormone (1, 2). The binding of ligands to these receptors is the initial step in a complex series of events culminating in an interaction of the ligand-bound receptor with the transcription machinery and modulation of gene expression. These receptor proteins exhibit four distinct properties required to exert their actions: hormone binding, multimeric complex formation, sequence specific DNA binding, and transcriptional modulation. The currently proposed schematic structure of these receptor proteins (shown in Fig. 1 for the estrogen receptor), based on sequence similarities and deletion analyses (summarized in Ref. 2), suggests that these proteins fold into at least three separate structural and functional domains: (i) an N-terminal domain having a highly variable length and amino acid sequence and believed to mediate much of the transcriptional enhancement activity of the

protein, (ii) a highly conserved central domain of ~80 amino acids involved in DNA binding, and (iii) a less well conserved C-terminal domain of ~250 amino acids that is involved in ligand binding.

The C-terminal hormone-binding domain (HBD)¹ of the receptor is of particular interest because, in addition to its ligand binding activity, it appears to contain many of the regulatory functions of the protein. Chimeric constructs containing fusions of fragments of the estrogen receptor with unrelated proteins such as the *myc* oncogene product, for example, display hormonal regulation of the activity of the fused gene products (3). This suggests that, even when removed from its normal environment, the HBD is not only capable of specific ligand binding, but may also retain the capacity to undergo the conformational changes that normally regulate the function of the receptor.

The nuclear receptor superfamily proteins are thought to form dimers. This property has at least two functional roles: most of the proteins in the family are thought to bind DNA as dimers, and dimer formation allows cooperative ligand binding, thereby narrowing the ligand concentration range required for full biological effect. The nature of the dimer interface of the full-length receptor protein is not established. The HBD is thought to play a role in dimerization, since mutations of residues within the estrogen receptor HBD have been shown to inhibit dimer formation (4). The isolated estrogen receptor DNA-binding domain has been shown to dimerize in the presence of DNA, suggesting that some of the dimerization interface resides within this portion of the protein; the isolated DNA-binding domain, however, is monomeric in solution (5, 6). It is not known how much of the interprotein interaction involved in dimer formation is actually contained within the HBD and whether the isolated HBD is fully capable of cooperative ligand binding.

Characterization of the estrogen receptor HBD has been limited by the difficulty of obtaining sufficient amounts of the peptide. In order to obtain preparations of the estrogen receptor HBD for more detailed study, several groups have attempted to express peptides containing the HBD in heterologous systems. Expression of the estrogen receptor HBD in yeast and bacteria has generally resulted in low yields of protein: ≤ 1 mg/liter (7, 8). Recently, Seielstad *et al.* (9) reported expression of an isolated HBD fragment in high yields in *Escherichia coli*; however, the protein produced using this system was insoluble, necessitating the use of urea during purification and characterization.

We describe herein a system which yields high level expression of soluble human estrogen receptor HBD in *E. coli*. The HBD peptide is produced as a fusion protein with the *E. coli*

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¹ The abbreviations used are: HBD, hormone-binding domain; AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride; MBP, maltose-binding protein; RAR, retinoic acid receptor, RXR, retinoid-X-receptor; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; FPLC, fast protein liquid chromatography.

maltose-binding protein at levels of ~10% of the total cell protein, and the fusion protein can be chemically cleaved to afford micromole quantities of the HBD peptide. We have characterized the cooperativity of estradiol binding and have examined the kinetics of dimer dissociation in solution.

MATERIALS AND METHODS

Supplies—Restriction endonucleases and other enzymes used for DNA manipulation were obtained from Boehringer Mannheim, New England Biolabs, Inc., Stratagene Cloning Systems (La Jolla, CA), or U. S. Biochemical Corp. Synthetic oligonucleotides were obtained from Operon Technologies (Alameda, CA). Bacterial growth media components were purchased from Difco (Detroit, MI); other reagents were obtained from Sigma. Tritiated estradiol was obtained from Amersham and DuPont NEN. The estrogen antagonist *trans*-4-hydroxytamoxifen was a gift from Dr. Dominique Salin-Drouin (Laboratoires Besins-Iscovesco) and ICI 182,780 was a gift from Dr. Alan Wakeling (ICI Pharmaceuticals).

Vector Construction—Unless otherwise noted, all DNA manipulations were carried out by standard techniques (10). A DNA fragment coding for the human estrogen receptor hormone-binding domain (amino acids 301–551) was generated by PCR from the HE0 estrogen receptor cDNA plasmid (11) using the following primers: 5' primer TCTAAGGAACAGCCTGGCCTTG, and 3' primer atcGaaTtcaCGCATGT-AGGCGGTGGCGCTCCAG; lowercase bases in the 3' PCR primer are mismatches that convert the codon for Pro-552 to a TGA termination codon and create an *Eco*RI site for subcloning. The PCR fragment was digested with *Eco*RI and subcloned into the pMAL-c2 vector (New England Biolabs) which had been digested with *Xmn*I and *Eco*RI. Following isolation of the insert-containing plasmid, the entire HBD coding region was sequenced to confirm the absence of errors introduced by PCR amplification. The presence of the cDNA mutation G400V (12) was verified by DNA sequencing; this mutation was reverted to wild-type using a PCR mutagenesis procedure (13), creating the plasmid pMAL-HBD1 (Fig. 1).

Protein products of pMAL-c2 derived plasmids consist of the maltose-binding protein fused to the desired protein with a linker peptide consisting of (Asn)₁₀-Leu-Gly-Ile-Glu-Arg; the terminal four residues of the peptide comprise a Factor X_a cleavage signal. Factor X_a hydrolysis of the expressed fusion protein, however, resulted in heterogeneous, largely inactive peptides; we therefore modified the linker region to generate the sequence Asn-Gly, which can be cleaved by hydroxylamine (14). Bases encoding residues Leu-Gly-Ile-Glu of the Factor X_a recognition sequence were mutated to Asn codons by site-directed mutagenesis using the unique site-elimination procedure (15) with the Transformer kit from Clontech. The coding region of the mutagenesis product was sequenced; the modified DNA was found to encode a linker peptide of (Asn)₁₄-Gly-Arg. This plasmid was designated pMAL-HBD2. Since N-terminal sequence analysis of the cleaved protein revealed that approximately 10–40% (the proportion varied in different preparations) of the protein was also cleaved between Asn-304 and Ser-305 of the HBD sequence (note doublet in Fig. 2, Lane 8), unique site-elimination was performed on pMAL-HBD2 to mutate Ser-305 → Glu, creating pMAL-HBD3. Fig. 1 shows the protein sequences surrounding the junction between the MBP and HBD for the three expression plasmids. The product of hydroxylamine cleavage of the fusion protein from the HBD2 and HBD3 constructs retains Gly-Arg from the linker, the latter of which corresponds to the naturally occurring Arg-300.

Protein Expression and Purification—Competent TOPP2 cells (Stratagene) were transformed with the expression plasmids pMAL-HBD1, pMAL-HBD2, or pMAL-HBD3. Cells containing the plasmid were grown in TB media in the presence of 100 µg/ml ampicillin to an OD₆₀₀ of ~1.7; protein expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.25 mM and cultures were grown overnight at ambient temperature (usually ~27 °C). Cells were resuspended in lysis buffer (50 mM Tris-HCl, 10 mM EDTA, 2 mM dithiothreitol, 1 mM AEBSF (Cal Biochem), pH 8.0, and 1 mg of lysozyme/g of cells). After ~1 h at ambient temperature, MgCl₂ was added to a final concentration of 120 mM and the lysate treated with DNase and RNase. The supernatant from a 40,000 × g centrifugation of the lysate was diluted 3-fold in TED buffer (20 mM Tris-HCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.3) and applied to a DEAE-cellulose column (Whatman). The flow-through from the DEAE-cellulose column was applied to an amylose resin column (New England Biolabs). After washing with 2–4 column volumes of TED containing

0.2 M NaCl, the fusion protein was eluted with 10 mM maltose in the same buffer.

The eluted protein was diluted 5-fold and applied to a DEAE-Sepharose column (Pharmacia). This column was washed with 5 column volumes of TED containing 0.05 M NaCl, and the protein was eluted with a linear NaCl gradient (0.05–0.2 M NaCl); the fusion protein eluted at 0.13–0.16 M NaCl. The fusion protein was then concentrated to ~20 mg/ml by precipitation with 60% ammonium sulfate and was digested for 60–72 h at ambient temperature with hydroxylamine (final concentration: 2 M hydroxylamine-HCl, 0.2 M Tris-HCl, pH 9.0). The cleaved HBD peptide was separated from the maltose-binding protein by Sephadex G-100 gel filtration chromatography. The final preparation of the purified HBD peptide was stable and could be stored at 4 °C or –70 °C for several months.

It should be noted that early preparations of the fusion protein and of the HBD had apparent estradiol binding stoichiometries significantly lower than 1:1, although the other properties of the protein were similar to those reported here. Addition of the DEAE-Sepharose chromatography step to the purification procedure for the fusion protein raised the stoichiometry of estradiol binding to close to 1:1, although this step had little effect on the apparent purity as assessed by SDS-PAGE. In contrast, FPLC Superdex-200 or gravity Sephadex G-100 gel filtration chromatography had no effect on the activity of the fusion protein or HBD samples.

Spectroscopy—All spectroscopy was performed at ambient temperature. Absorbance spectra were obtained using a Cary 1 spectrophotometer calibrated with K₃Fe(CN)₆ assuming $\epsilon_{420} = 1,020 \text{ (M cm)}^{-1}$. The concentration of purified MBP-HBD fusion protein and isolated HBD peptide were determined spectrophotometrically assuming $\epsilon_{280} = 89,365 \text{ (M cm)}^{-1}$ for the fusion protein and $23,745 \text{ (M cm)}^{-1}$ for the HBD peptide; these values are based on a composition of 11 tryptophan and 20 tyrosine residues (fusion protein) or 3 tryptophan and 5 tyrosine residues (HBD peptide) predicted from the cDNA sequence and on average extinction coefficients for tryptophan (5615 (M cm)^{-1}) and tyrosine (1380 (M cm)^{-1}) (16, 17). Concentrations of HBD determined spectrophotometrically agreed closely with those determined by the methods of Lowry *et al.* (18) and Bradford (19).

Circular dichroism spectra were obtained using a Jasco J-720 spectropolarimeter with a 0.05-cm path length cell and a band pass of 2 nm. Twelve scans were collected and averaged. Theoretical curve fitting to estimate secondary structure content was performed using the k2d program (20).

Analytical Gel Filtration—The apparent molecular weight of the fusion protein and HBD were determined using a Pharmacia FPLC system and a Superdex 200 HR 10/30 gel filtration column (running buffer 20 mM Tris-HCl, 1 mM EDTA, 200 mM NaCl, pH 7.3). The column was calibrated using blue dextran to determine the void volume and with the following standard proteins: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (69 kDa), ascorbate peroxidase (57.5 kDa), P450eryF (45.8 kDa), ovalbumin (43 kDa), MBP (40.4 kDa), rhodanese (33.3 kDa), chymotrypsinogen (25 kDa), ribonuclease A (13.7 kDa), and cytochrome c (12.4 kDa).

For kinetic experiments, equimolar amounts of the fusion protein and HBD peptide were mixed and incubated at ambient temperature (~25 °C). At various times aliquots were taken and subjected to FPLC gel filtration. For the experiments in the presence of ligand, the column was pre-equilibrated in the same running buffer with 50 nM of the relevant ligand, and 2 µM solutions of each protein pre-equilibrated overnight with 5 µM of the ligand. The integrated peak areas were corrected for extinction coefficient of the relevant protein species to determine the concentration of each species (*i.e.* fusion homodimer, HBD homodimer, or heterodimer) present at the time of injection (the relative amount of each species was assumed not to change during the chromatography). For experiments in the presence of ligand, the extinction coefficient of the protein was corrected for contributions of the bound ligand (assumed to be ~2,000 (M cm)⁻¹ for estradiol and ~15,000 (M cm)⁻¹ for 4-hydroxytamoxifen).

The rate constant for dissociation, *k*, was determined by least-squares nonlinear regression of the first-order rate equation,

$$D_t = (D_0 - D_f)e^{-kt} + D_f \quad (\text{Eq. 1})$$

where *D_t* is the concentration of one homodimer at time *t*, *D₀* is the initial concentration of homodimer, and *D_f* is the final concentration of homodimer after the rearrangement had gone to completion.

Protein Sequence Determination—Amino-terminal sequence data was obtained for purified cleaved protein by automated Edman degra-

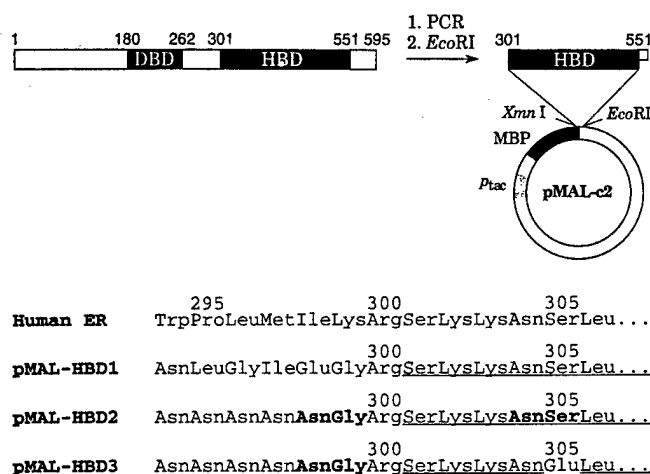


FIG. 1. Schematic structure of the estrogen receptor and construction of the human estrogen receptor HBD expression vectors. The schematic structure used the amino acid numbering for the human estrogen receptor. Amino acids 1–180 comprise the N-terminal domain, 180–262 comprise the DNA-binding domain, and 301–551 comprise the hormone-binding domain. A fragment of the cDNA corresponding to the HBD was amplified by PCR and subcloned into pMAL-c2. This original plasmid (pMAL-HBD1) was then converted to the other constructs by site-directed mutagenesis. Partial protein sequences for the human estrogen receptor and for each fusion protein near its cleavage site are shown. For each expressed protein the coding sequence derived from the estrogen receptor is *underlined*, and the site(s) cleaved by hydroxylamine are shown in *bold*.

dation performed by Dr. Agnes Henshen-Edman (University of California, Irvine).

Radioceptor Assay—Dilutions of the HBD peptide were incubated overnight with various concentrations of [6,7-³H]estradiol at 4 °C in TED buffer including 0.2 M NaCl and 1 mg/ml porcine gelatin; bound and unbound steroids were separated using dextran-coated charcoal (0.625% charcoal, 0.125% dextran) in the same buffer without gelatin. In all experiments using purified and partially purified protein, the binding of radioactive estradiol in the presence of a 100-fold excess of unlabeled estradiol was equivalent to the nonspecific binding observed in the absence of any added HBD protein. The presence of a carrier protein in both the ligand and protein buffers was found to be necessary to obtain reproducible results; porcine gelatin (1 mg/ml), bovine γ -globulin (4 mg/ml), or bovine serum albumin (4 mg/ml) gave similar results.

The data for bound and free steroid were directly fitted to the Hill equation (21),

$$[B] = \frac{B_{\max}[F]^n}{(F_{0.5})^n + [F]^n} \quad (\text{Eq. 2})$$

using least squares nonlinear regression analysis to estimate the $F_{0.5}$ (or K_d when $n = 1$), B_{\max} , and n (Hill coefficient). The parameters obtained from the nonlinear regression analysis were used to generate theoretical curves, which are shown in the Scatchard (22) plots in Figs. 3 and 8.

For competitive ligand binding experiments, the HBD peptide was incubated with various concentrations of ligand in the presence of a constant amount of tritiated estradiol. The amount of bound [³H]estradiol is presented as a percent of that bound in the absence of competitor ligand.

RESULTS

Expression and Isolation of the HBD Peptide—The pMAL-c2 expression system produces the protein of interest as a fusion with the *E. coli* maltose-binding protein. The MBP is well expressed, stable, and can be purified by amylose affinity chromatography. The linker peptide of the fusion protein is designed to be cleavable by the endoproteinase Factor X_a to release the fused protein without additional N-terminal residues. The construction of vectors for expressing the MBP-HBD peptide is shown schematically in Fig. 1, and described under "Materials and Methods."

Induction of protein expression from each of the pMAL-HBD

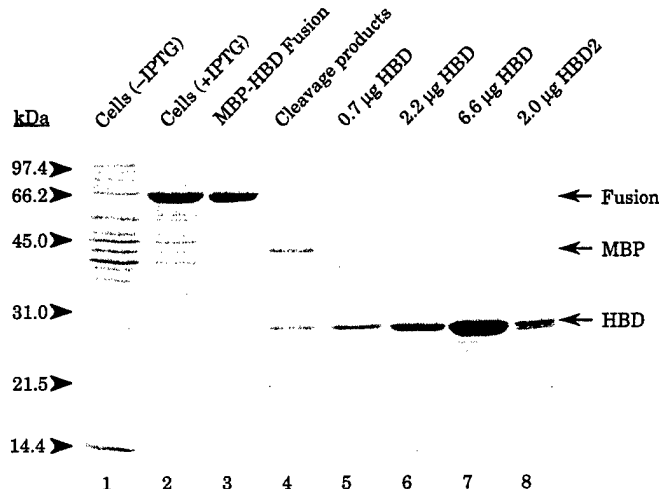


FIG. 2. SDS-PAGE analysis of HBD peptides during purification. A 12% polyacrylamide gel stained with Coomassie Blue is shown: Lane 1, whole cells from an overnight culture of TOPP2 *E. coli* harboring the pMAL-HBD3 expression plasmid; Lane 2, whole cells from an overnight culture following induction with 0.25 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG); Lane 3, partially purified fusion protein; Lane 4, the products of the hydroxylamine cleavage reaction; Lanes 5–7, 0.7–6.6 μ g of purified HBD peptide. Lane 8, purified hydroxylamine cleavage product from pMAL-HBD2; note doublet band due to heterogeneous cleavage. With the exception of Lane 8, this gel represents protein produced from pMAL-HBD3; Lanes 1–3 appeared similar using all three pMAL-HBD constructs.

plasmids with isopropyl-1-thio- β -D-galactopyranoside produced significant quantities of fusion protein, estimated to comprise approximately 10% of the cell protein (Fig. 2, Lane 2). Essentially all of this fusion protein appeared to be soluble in the cells. Attempts to purify the MBP-HBD fusion from crude cell homogenates by amylose affinity chromatography, however, were unsuccessful; binding of the fusion protein from the crude extract to the affinity column was incomplete, and the eluted protein was not highly purified. For this reason, the cell lysate was first subjected to anion exchange chromatography. The partially purified fusion protein in the unbound fraction from the anion exchange column bound the amylose column nearly quantitatively, and upon elution from the column exhibited only minor contaminants (Fig. 2, Lane 3).

Enzymatic cleavage of the partially purified MBP-HBD1 fusion protein with either Factor X_a or trypsin resulted in degradation to heterogeneous products that exhibited markedly reduced ability to bind estradiol (data not shown). Chemical cleavage methods were then tested as alternatives to proteolytic digestion. Hydroxylamine preferentially hydrolyzes the peptide bond of Asn-Gly sequences, although solvent-exposed Asn-Xaa sequences are hydrolyzed at lower rates (14). The HBD peptide does not contain any Asn-Gly sequences and would therefore be expected to be relatively resistant to hydroxylamine. Modifications to the original construct resulted in the expression plasmid pMAL-HBD3 (Fig. 1); the fusion protein from this plasmid appeared to cleave quantitatively cleavage at the correct site, yielding a final peptide with Gly followed by amino acids 300–551 (S305E) of the HBD (see Fig. 2, Lane 4, for the results of the cleavage reaction).

The HBD peptide was separated from the MBP by gel filtration chromatography. Analysis of a final preparation of the HBD peptide by SDS-PAGE is shown in Fig. 2, Lanes 5–7. The HBD peptide has an apparent mass similar to the predicted ~29 kDa. Based on the staining intensities observed the overall purity is estimated to be >90%; the minor impurity band visible at the highest concentration of HBD peptide is estimated to comprise ~1–2% of the total protein (based on densi-

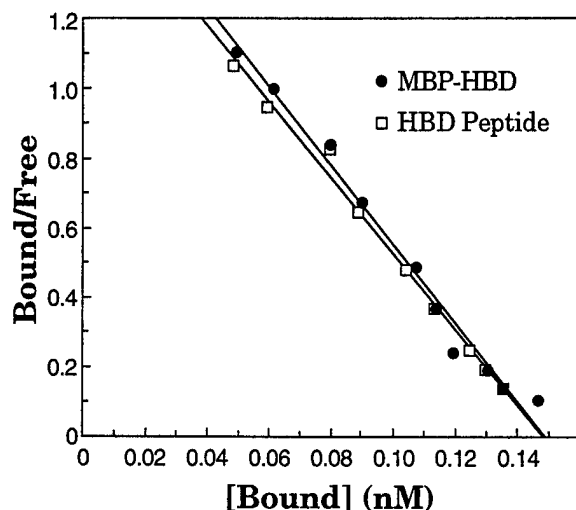


FIG. 3. Scatchard analysis for estradiol binding to the MBP-HBD peptide fusion protein and purified HBD peptide. A radio-receptor assay was performed using 0.15 nM MBP-HBD fusion protein or 0.15 nM purified HBD peptide. Both nonlinear regression analysis of the bound and free data, and linear regression of the bound/free versus bound Scatchard transformation data yield apparent K_d values of 0.1 nM and apparent B_{max} values corresponding to 0.98 mol of estradiol/mol of protein for both the fusion protein and the HBD peptide.

tometry of the Coomassie-stained gel), and is a degradation product of the HBD peptide. The final yield was typically ~10 mg of purified HBD peptide per liter of bacterial culture for several preparations; this corresponds to approximately a 40% yield from the total amount of fusion protein estimated to be present in the cells.

Ligand Binding—Both the purified HBD peptide and the MBP-HBD fusion protein were assayed for their ability to bind estradiol. Fig. 3 shows the results of typical Scatchard analyses of [3 H]estradiol binding using low concentrations (0.15 nM) for each protein. The K_d values obtained (0.1 nM) are similar to those reported for the full-length estrogen receptor protein obtained from human cells (*cf.* Ref. 8) indicating that the estrogen binding properties of the HBD are not affected either by isolation from other parts of the receptor or by fusion to the maltose-binding protein. The B_{max} values determined in this experiment corresponded to a binding stoichiometry of ~0.98 mol of estradiol bound per mol of HBD peptide. No significant changes in stoichiometry have been observed following the hydroxylamine cleavage step, suggesting that little denaturation occurs under the conditions of the cleavage procedure.

Ligand Discrimination by the HBD Peptides—The ability of the HBD peptide to discriminate between different ligands was assessed by competitive binding assays using [3 H]estradiol, and the competition binding curves are presented in Fig. 4. The ligand discrimination profile exhibited by the isolated HBD peptide is generally similar to that reported for the full-length native receptor (8). The weak agonist estrone exhibited about 10-fold lower affinity than estradiol, whereas testosterone (and progesterone, data not shown) did not appear to compete significantly even at concentrations 35,000-fold greater than those used for estradiol. The steroidal antagonist ICI 182,780 bound with an affinity intermediate between that of estradiol and estrone. Two non-steroidal antagonists, *trans*-tamoxifen and *trans*-4-hydroxytamoxifen, were tested and also found to be effective competitors. The *trans*-4-hydroxytamoxifen had a slightly lower affinity and a steeper slope on the semi-log plot than that of estradiol, similar to observations previously reported by Sasson and Notides (23) for the full-length calf uterine estrogen receptor. The non-parallel competition curve for 4-hydroxytamoxifen was interpreted by Sasson and Notides

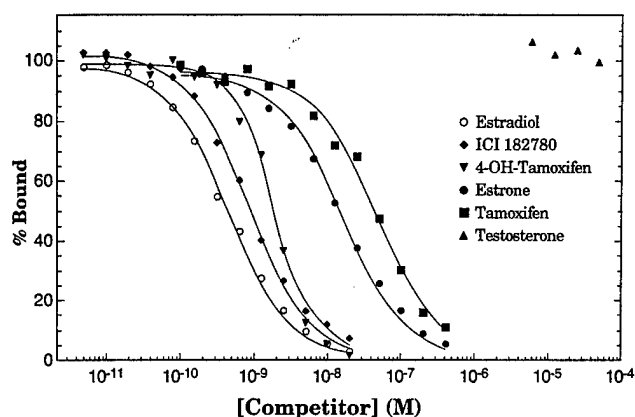


FIG. 4. Competition ligand binding curves for the purified HBD peptide. Assays were performed using 1 nM purified peptide and 1.1 nM [3 H]estradiol. The data shown represent the average of two experiments performed in duplicate.

(23) to indicate that estradiol and 4-hydroxytamoxifen bind the receptor differently, and that 4-hydroxytamoxifen binding to one site in the dimer induced the dissociation of estradiol from the other site. Our observation of the non-parallel competition of estradiol by 4-hydroxytamoxifen suggests that the structural features required for this differential binding of the two ligands are retained by the isolated HBD.

Circular Dichroism Spectra of the Purified HBD Peptide—Far ultraviolet circular dichroism was used to investigate the peptide backbone secondary structure (Fig. 5). The spectrum exhibits a maximum near 195 nm, and minima at 208 and 222–223 nm. Curve-fitting to the data using the k2d computer program (20) suggests a composition of ~60% α -helix and ~7% β -strand. Both secondary structure prediction methods (24, 25) and the known crystal structure of the related retinoid-X-receptor- α (RXR- α), retinoic acid receptor- γ (RAR- γ), and thyroid hormone receptor- α HBDs (26–28) predict a largely helical fold, and the latter proteins have similar helical character (60–65%) and similar amounts of β -strand (5–10%). CD spectra were also recorded for the HBD in the presence of equimolar amounts of the ligands estradiol and *trans*-4-hydroxytamoxifen; these spectra were essentially identical to the spectrum in the absence of ligand (Fig. 5), suggesting that any conformational changes induced by ligand binding do not involve significant changes in overall secondary structure.

Size Exclusion Chromatography—The hormone-binding domain is thought to contain a region at least partially responsible for dimerization of the full-length protein (2, 4, 29). We used gel-filtration chromatography to determine whether the fusion protein and HBD peptide formed dimers (or larger multimers) in solution. When the cleaved HBD peptide was separated from the MBP by Sephadex G-100 gel filtration chromatography during the purification procedure, the HBD peptide was found to elute near the void volume of the column, well ahead of the ~40-kDa MBP. This suggested that the HBD peptide (monomer ~29 kDa) exists as a multimeric complex under the conditions used for the preparative G-100 gel filtration (~100 μ M initial peptide concentration). This was examined further using analytical FPLC Superdex-200 gel filtration chromatography. When run independently on the Superdex column, both the fusion protein and HBD peptide migrated as single peaks. The apparent M_r of the fusion protein (156,000) was similar to that predicted for a dimer (142,000). Because the isolated MBP is known to be monomeric and migrates close to its predicted size of 40,000, this suggests that fusion protein dimerization is mediated by the HBD peptide fragment. The HBD peptide alone migrated with an apparent M_r of 47,000, intermediate

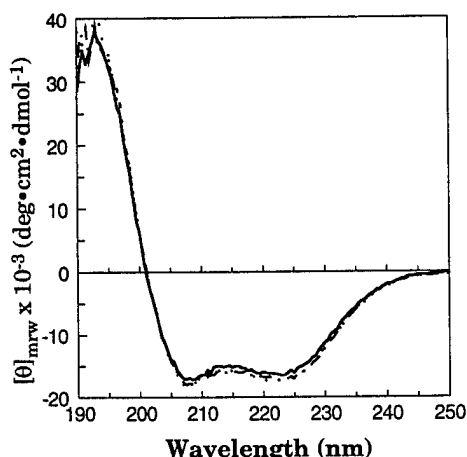


FIG. 5. Far-ultraviolet circular dichroism spectra for the HBD peptide. The concentration of HBD peptide used was 10 μ M in 20 mM Tris buffer (pH 7.4). The solid line represents the HBD peptide alone; the dotted and dashed lines represent the HBD peptide in the presence of equimolar amounts of estradiol and *trans*-4-hydroxytamoxifen, respectively.

between that expected for a dimer (58,000) or monomer (29,000). To determine whether the HBD peptide exists as a dimer, equimolar amounts of the fusion protein and HBD peptide were mixed, allowed to equilibrate for 24 h, and then subjected to gel filtration. Under these conditions, a third peak appeared at 99,000 (Fig. 6), a position corresponding to a heterodimer of the fusion protein (72,000) and the HBD peptide (29,000). Integration of the peak areas (corrected for the extinction coefficients of fusion homodimer, heterodimer, and HBD peptide homodimer) yielded a ratio of 1:2:1, confirming the identity of the intermediate peak as a heterodimer formed between fusion protein and HBD peptide monomers. The fact that a single additional peak was formed also suggests that both fusion protein and HBD peptide predominantly form dimers, but not larger multimers, in solution. No significant changes were observed in the migration of the HBD peptide or fusion protein on the Superdex column using concentrations ranging from 0.5 to 10 μ M. This suggested that for both the fusion protein and HBD peptide, the majority of the protein was present as dimer under these conditions.

In order to form the heterodimer, these homodimers must dissociate, and this dissociation probably constitutes the rate-limiting step for heterodimer formation. The fusion protein and HBD peptide were therefore subjected to gel filtration at various times after mixing. A plot of homodimer concentration (determined from peak area) versus time after mixing fits a first-order exponential (Fig. 7); the rate constant for dissociation was determined to be $0.60 \pm 0.14 \text{ h}^{-1}$, corresponding to a half-life of 1.2 h.

We also studied the effects of ligand binding on the dimer dissociation. For these experiments the protein was pre-equilibrated with saturating amounts of estradiol or 4-hydroxytamoxifen. These results, also shown in Fig. 7, are summarized in Table I. The data suggest that presence of estradiol significantly decreased the rate of dissociation of the receptor dimer. The antagonist ligand *trans*-4-hydroxytamoxifen was also found to significantly decrease the rate of dissociation, to an even greater degree than estradiol.

Effect of HBD Peptide Concentration on Estradiol Binding—The full-length native estrogen receptor protein has been shown to exhibit positive cooperativity (30, 31). We measured [^3H]estradiol binding using several different concentrations of the HBD peptide to determine whether this aspect of the functional nature of the dimer was retained; the data were analyzed

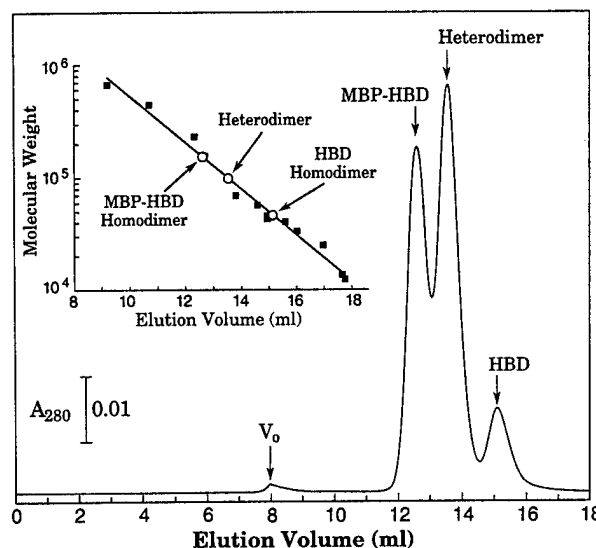


FIG. 6. FPLC chromatogram of a mixture of MBP-HBD fusion protein and HBD peptide. The peaks labeled "MBP-HBD" and "HBD" co-migrate with purified samples of the corresponding protein run separately; the peak labeled "Heterodimer" only appears when the two proteins are mixed prior to chromatography. The experiment shown was performed in the absence of ligand; no change in migration was observed in the presence of estradiol or 4-hydroxytamoxifen (data not shown). The inset shows the migration positions of the three estrogen receptor-derived protein complexes in comparison with the standard proteins used to calibrate the column (see "Materials and Methods").

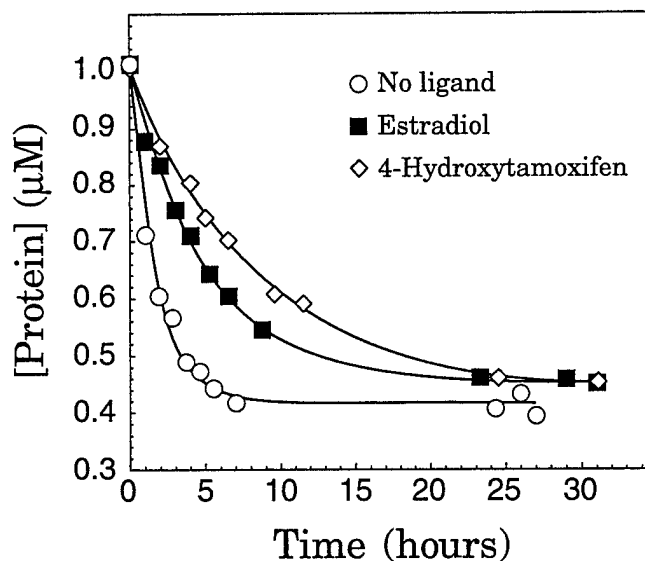


FIG. 7. Kinetics of homodimer dissociation. The homodimer concentration (determined from the peak area corrected for protein extinction coefficient) was plotted against time after mixing. For clarity, only the fusion protein homodimer concentration is shown; HBD peptide homodimer yielded similar kinetics. The curves represent nonlinear regression fits to an exponential rate equation. \circ , absence of ligand; \blacksquare , presence of estradiol; \diamond , presence of 4-hydroxytamoxifen.

by least-squares nonlinear regression as described under "Materials and Methods." Fig. 8 shows the results of a typical experiment at several concentrations of the HBD peptide. At the lowest concentration shown (2 nM HBD peptide) the best fit to the data has a Hill coefficient of 1.0, yielding a straight line on the Scatchard plot similar to that presented in Fig. 3 in which 0.15 nM HBD peptide was used. In contrast, at the higher concentrations (4–15 nM HBD peptide) the data exhibit convex curvature indicative of positive cooperativity. The inset in Fig. 8 shows a Hill plot of the data from the highest concentration of the HBD peptide; the line determined from this set of data

has a slope (Hill coefficient) $n = 1.54$, indicating positive cooperativity. In a series of these experiments, non-cooperative estradiol binding was observed when the peptide concentration was less than ~ 2 nM, and maximal cooperativity was observed at concentrations greater than ~ 3 nM. The observed $F_{0.5}$ values increased from ~ 0.1 to ~ 0.5 nM over the same HBD peptide concentration range. The maximal Hill coefficient observed for the HBD peptide ($n = 1.5$ – 1.6) is similar to the value of ~ 1.6 reported for the full-length receptor (31). Moreover, the concentration range over which ligand binding to the isolated HBD peptide changes from non-cooperative to cooperative behavior (~ 2 – 3 nM) is also similar to the range of 1 – 7 nM over which the full-length human receptor begins to exhibit cooperativity (31). These results suggest that most or all of the structural features required for cooperative ligand binding interactions are retained in the HBD peptide fragment.

DISCUSSION

We have expressed, purified in high yield, and carried out initial characterization of the hormone-binding domain of the human estrogen receptor. The yield of soluble HBD peptide obtained (~ 10 mg/liter of culture) is significantly greater than previously reported, and represents amounts of protein that can be used for a variety of biophysical studies. Previous attempts at HBD peptide expression in *E. coli* (7, 8) used constructs coding for estrogen receptor peptides comprising amino acid residues 240–595, and therefore sequences of more than 350 amino acids. Moreover, these expressed peptides were fused to either a peptide from β -galactosidase (7) or to Protein A (8), and the HBD peptide products were not reported to be either purified or cleaved from the fusion proteins. Recently, expression of an HBD peptide fragment (amino acids 282–595) in high yield in insoluble form from a T7 RNA polymerase expression plasmid was reported (9). This protein, however,

required 1 M urea for solubilization and 5 M urea and estradiol for purification; moreover, the preparation was heterogeneous as a result of proteolysis at positions 569 or 571.²

The expression system we describe herein produces an active fragment of the estrogen receptor comprising only 253 amino acids (positions 300–551, with an additional Gly at the amino terminus and Ser-305 mutated to Glu) following purification and cleavage.³ As discussed in more detail below, the estrogen receptor HBD peptide exhibits ligand binding and ligand discrimination properties comparable to the full-length protein, suggesting that the changes incorporated do not grossly alter the structure of this domain of the receptor. The reduced size of our construct should simplify interpretation of biophysical data regarding the expressed protein. In addition, this peptide is expressed in soluble form at high levels and does not require the use of urea nor estradiol in the purification procedure. It is not clear whether the high level of expression we observe is due to the composition of the estrogen receptor-derived peptide, to the efficiently expressed maltose-binding protein used as a leader, or to a combination of the two.

Properties of the HBD Peptide—The HBD peptides exhibited both high affinity estradiol binding ($K_d \sim 0.2$ nM), and ligand discrimination similar to that of the full-length receptor. The ligand binding observed is consistent with a single ligand interacting with a single HBD peptide monomer. The CD spectrum suggests that the HBD peptide is largely helical, as expected from both secondary structure prediction and from the crystal structures of the related ($\sim 25\%$ sequence identity) RAR- γ (26) and RXR- α (27) and thyroid hormone receptor- α HBD (28) peptides. The observation that the CD spectrum is essentially unchanged in the presence of both agonist ligand estradiol and the non-steroidal antagonist ligand *trans*-4-hydroxytamoxifen indicates that the overall amount of secondary structure is unaffected by the conformational changes that occur upon interaction with either type of ligand. Comparison

TABLE I
Effect of ligand binding on hormone-binding domain dimer dissociation

Ligand	Rate constant for dissociation ^a	$t_{1/2}$ ^a	Relative effect
	h	h	fold change
None	0.60 ± 0.14	1.2 ± 0.3	
Estradiol	0.19 ± 0.03^b	3.7 ± 0.6^b	3.0
4-Hydroxytamoxifen	0.14 ± 0.03^b	5.3 ± 1.4^b	4.4

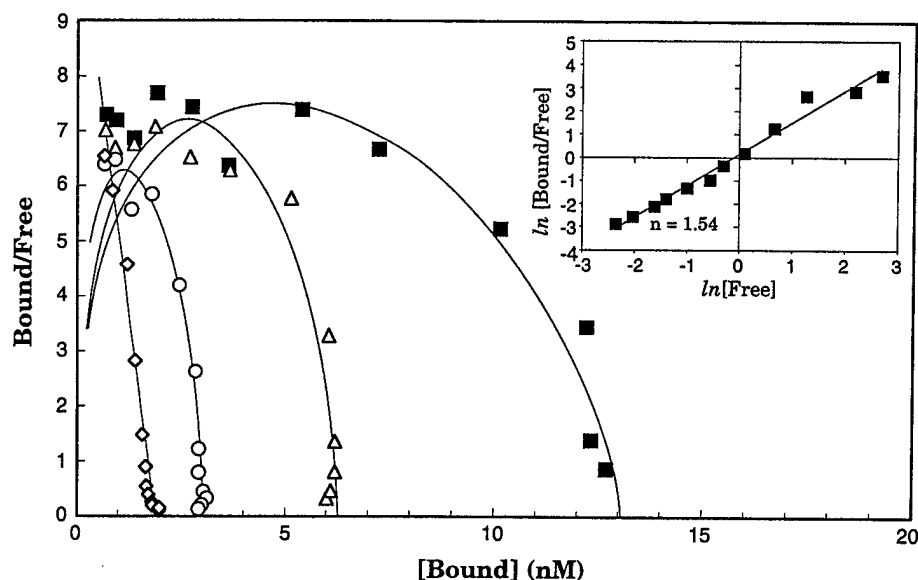
^a Values are presented as mean \pm S.D.

^b Significantly different from control, $p < 0.001$.

² We have also found that whole cells expressing constructs intended to produce protein extending to the C terminus of the estrogen receptor at position 595 contain variously sized estrogen receptor protein products, with the major product ending near position 567 based on SDS-PAGE analysis. Constructs ending at amino acid 567 or 551 appear to yield significantly higher levels of expressed MBP-HBD fusion protein than do constructs intended to extend to position 595.

³ For the purpose of comparison, residues 225–462 were visible in the RXR- α HBD crystal structure (26); these are thought to correspond to residues 309–551 of the estrogen receptor (32).

FIG. 8. Scatchard analyses for estradiol binding at varying concentrations of the HBD peptide. Four concentrations of the HBD peptide from a typical experiment are depicted showing development of positive cooperativity of estradiol binding. The lines represent curves generated by fits of the data to the Hill equation. ■, 16 nM protein (Hill coefficient = 1.54); △, 8 nM protein (Hill coefficient = 1.72); ○, 4 nM protein (Hill coefficient = 1.58); and ◇, 2 nM protein (Hill coefficient = 1.0). The inset shows a Hill plot of the data for 16 nM HBD peptide. The variation between the stated concentration of HBD peptide and the apparent B_{max} is due to absorbing species (probably oxidized dithiothreitol and small amounts of denatured protein) that do not exhibit binding.



of the monomeric agonist-complexed RAR- γ and dimeric ligand-free RXR- α structures shows that the major conformational changes are confined to movements of one loop and of the α -helix at the C terminus of the HBD peptide (27, 32). Our CD data provide experimental support for a similar model for the estrogen receptor, and further suggest that alterations in dimer interactions as a result of ligand binding also do not involve major changes in secondary structure.

Dimerization and Cooperativity of Ligand Binding—The estrogen receptor is thought to bind to DNA as a dimer (2, 4, 29). Mutations within the C-terminal region of the HBD peptide (positions 507–518 of the mouse receptor, corresponding to 503–514 of the human estrogen receptor) have been shown to disrupt dimerization (4, 33). In addition, the RXR- α HBD peptide crystallized as a dimer (26). On the other hand, the HBD peptides from two other related proteins, RAR- γ (27) and thyroid hormone receptor- α (28), crystallized as monomers. Although the isolated estrogen receptor DNA-binding domain is monomeric in solution (5), it binds DNA as a dimer (6), raising the question as to whether interactions involving residues in the HBD peptide alone are sufficient to allow stable dimer formation. At the high concentrations used for the preparative G-100 column ($\sim 100 \mu\text{M}$) and the intermediate concentrations used for the Superdex S-200 chromatography (0.5–10 μM), both the fusion protein and HBD peptide migrated at apparent sizes significantly greater than those predicted for their respective monomers, and a mixture of fusion protein and HBD peptide resulted in the appearance of third peak corresponding to a heterodimer both in the presence and absence of ligand. Our results thus suggest that the isolated HBD peptide contains the amino acid sequences sufficient for dimerization, and furthermore, that dimer formation does not require ligand binding.

Dimer formation was also implied by the finding that the HBD peptide exhibited positively cooperative estradiol binding, with a maximal Hill coefficient of ~ 1.6 . Cooperativity of estradiol binding to the estrogen receptor has been most extensively studied using the protein from calf uterine cytosol (30, 34), and the maximal Hill coefficient cited in these reports is ~ 1.6 at a concentration of $\sim 5 \text{ nM}$ receptor. Using recombinant full-length human estrogen receptor expressed in Sf9 insect cells, Obourn *et al.* (31) also found a maximal Hill coefficient of ~ 1.6 ; their data suggest that at receptor concentrations below $\sim 1 \text{ nM}$, the receptor does not exhibit cooperativity, while maximal cooperativity is observed at 10–20 nM receptor concentration. We observe a transition from non-cooperative to cooperative behavior for the isolated HBD fragment in the range from 2 to 3 nM, suggesting that the HBD peptide undergoes the conformational changes required for cooperativity in a manner comparable to the full-length protein. One interpretation of these results is that dimer formation occurs over this peptide concentration range in the presence of estradiol. Thus, at concentrations below $\sim 2 \text{ nM}$, the HBD peptide exists as a monomer, while at concentrations above 3 nM, the HBD peptide is predominantly present as a dimer which exhibits a cooperative interaction with estradiol.

The ligand-bound RAR and thyroid hormone receptor structures were determined for monomeric proteins, and therefore do not allow conclusions to be drawn as to the structural effect of ligand binding on dimer interactions. The cooperativity data suggest that conformational changes in one subunit of the dimer affect the other subunit. In order to examine the effect of ligand binding on dimer interactions more directly, we took

advantage of the significant difference in size between the fusion protein and HBD peptide. We used size-exclusion chromatography to examine the exchange from homodimers to heterodimers of one fusion and one HBD monomer. In the absence of ligand, the dissociation (presumed to be the rate-limiting step in the exchange process) was slow, with a half-life of $\sim 1.2 \text{ h}$. In the presence of estradiol the half-life increased significantly by a factor of ~ 3 -fold. The change in dissociation kinetics suggests that ligand binding results in a conformational change in the HBD that affects the dimer interface. The fact that the antagonist 4-hydroxytamoxifen also decreases the rate of dissociation implies that it induces conformational changes in the dimer interface similar to those of estradiol. The increase in half-life suggests that one role of ligand binding is to increase the kinetic stability of the estrogen receptor dimer-ligand complex. One effect of this may be to increase the time available for the receptor dimer to interact with the other proteins of the transcription initiation complex.

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REFERENCES

- Evans, R. M. (1988) *Science* **240**, 889–895
- Tsai, M. J., and O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63**, 451–486
- Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. (1989) *Nature* **340**, 66–68
- Fawell, S. E., Lees, J. A., White, R., and Parker, M. G. (1990) *Cell* **60**, 953–962
- Schwabe, J. W. R., Neuhaus, D., and Rhodes, D. (1990) *Nature* **348**, 458–461
- Schwabe, J. W. R., Chapman, L., Finch, J. T., and Rhodes, D. (1993) *Cell* **75**, 567–578
- Ahrens, H., Schuh, T. J., Rainish, B. L., Furlow, J. D., Gorski, J., and Mueller, G. C. (1992) *Receptor* **2**, 77–92
- Woo, C. H., Nilsson, G. M., Heierson, A., McDonnell, D. P., and Katzenellenbogen, B. S. (1992) *Mol. Endocrinol.* **6**, 861–869
- Seielstad, D. A., Carlson, K. E., Katzenellenbogen, J. A., Kushner, P. J., and Greene, G. L. (1995) *Mol. Endocrinol.* **9**, 647–658
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Kumar, V., Green, S., Staub, A., and Chambon, P. (1986) *EMBO J.* **5**, 2231–2236
- Tora, L., Mullick, A., Metzger, D., Ponglikitmongkol, M., Park, I., and Chambon, P. (1989) *EMBO J.* **8**, 1981–1986
- Nelson, R. M., and Long, G. L. (1989) *Anal. Biochem.* **180**, 147–151
- Bornstein, P., and Balian, G. (1977) *Methods Enzymol.* **47**, 132–145
- Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem.* **200**, 81–88
- Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182**, 319–326
- Mach, H., Middaugh, C. R., and Lewis, R. V. (1992) *Anal. Biochem.* **200**, 74–80
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Andrade, M. A., Chac-n, P., Merelo, J. J., and Morán, F. (1993) *Protein Eng.* **6**, 383–390
- Hill, A. V. (1913) *Biochem. J.* **7**, 471
- Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
- Sasson, S., and Notides, A. C. (1988) *Mol. Endocrinol.* **2**, 307–312
- Chou, P. Y., and Fasman, G. D. (1974) *Biochemistry* **13**, 222–245
- Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) *J. Mol. Biol.* **120**, 97–120
- Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375**, 377–382
- Renaud, J. P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **378**, 681–689
- Wagner, R. L., Apriletti, J. W., McGrath, M. E., West, B., Baxter, J. D., and Fletterick, R. J. (1995) *Nature* **378**, 690–697
- Kumar, V., and Chambon, P. (1988) *Cell* **55**, 145–156
- Notides, A. C., Lerner, N., and Hamilton, D. E. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78**, 4926–4930
- Obourn, J. D., Koszewski, N. J., and Notides, A. C. (1993) *Biochemistry* **32**, 6229–6236
- Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D., and Gronemeyer, H. (1996) *Nature Struct. Biol.* **3**, 87–94
- Lees, J. A., Fawell, S. E., White, R., and Parker, M. G. (1990) *Mol. Cell. Biol.* **10**, 5529–5531
- Schwartz, J. A., and Skafar, D. F. (1994) *Biochemistry* **33**, 13267–13273

**ESTROGEN RECEPTOR HORMONE BINDING DOMAIN
COOPERATIVITY AND DIMERIZATION**

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The estrogen receptor, a ligand-activated transcription factor, has been implicated in the control of genes involved in differentiation and cell growth in both normal tissues and breast cancer. As part of its normal functioning the estrogen receptor dimerizes and exhibits cooperative ligand binding. The regions of the molecule responsible for dimerization have not been precisely defined, but residues within a C-terminal portion of the protein, the hormone binding domain (HBD), are thought to be involved. In order to assess the role of the HBD in dimerization and to characterize its ligand binding properties, we have expressed human estrogen receptor HBD fragments in *Escherichia coli*.

We used a modified pMAL-c2 expression plasmid (New England Biolabs) for the protein expression, which yields the peptide of interest fused to maltose-binding-protein; the fusion protein was then chemically cleaved to release the isolated HBD peptide (Brandt & Vickery (1997) *J. Biol. Chem.* **272**: 4843-4849). Coding sequences for HBD fragments with different starting positions (from 300 to 312, with all constructs ending with residue 551) were generated by PCR and sub-cloned into the pMAL plasmid. Constructs beginning at estrogen receptor position 306 or earlier resulted in stable peptides, while a construct beginning with position 310 exhibited reduced stability, and peptides beginning at later positions failed to express detectable levels of protein. For stable constructs, both the fusion protein and the isolated HBD exhibited high affinity binding for estradiol ($K_d \sim 0.2$ nM) and differential recognition of ligands similar to the full-length receptor. The heterologous expression system yields 10-20 mg of purified HBD peptide per liter of culture.

We have begun spectroscopic characterization of the HBD. The ultraviolet circular dichroism spectrum suggests that the HBD peptide contains significant amounts of α -helix (~60%) and some β -strand (~7%). These proportions are very similar to those observed for the recently published crystal structure of the related retinoid-X receptor HBD, suggesting a similar overall fold to the estrogen

receptor HBD. Ligand binding to the HBD peptide resulted in little, if any, change in secondary structural content. Fluorescence spectroscopic analysis of the HBD peptides gave excitation and emission spectra typical of tryptophan; the emission maximum occurs at 335 nm, suggesting that one or more of the tryptophan residues is exposed to solvent. Binding of estradiol had little effect on the fluorescence spectrum; however, binding of the antagonist ligand *trans*-4-hydroxytamoxifen markedly decreased the tryptophan fluorescence intensity.

The cooperative ligand binding exhibited by the full-length protein requires the formation of a multimeric complex. Complex formation for the HBD was assessed by size exclusion chromatography, and the results suggested that both the fusion protein and isolated HBD peptide are predominantly present as dimers in solution. The rate of HBD dimer dissociation was measured and found have a half-life of ~1 to ~3 hours (depending on the HBD peptide N-terminus sequence), which ligand binding increased ~3-fold (estradiol) to ~4-fold (4-hydroxytamoxifen). At peptide concentrations >10 nM, the isolated HBD peptides displayed cooperative estradiol binding with a Hill coefficient of ~1.6, suggesting that the HBD exhibits cooperative ligand binding characteristics similar to the full length receptor. Analysis of stable peptides beginning at different positions exhibited small quantitative, but not qualitative differences in cooperativity.

These results suggest that the isolated estrogen receptor HBD dimerizes and undergoes conformational changes in a manner comparable to the full-length protein. The N-terminal region of the HBD appears to play an important role in its stability, since relatively small differences in residues 300-310 (minor truncations or point mutations) have significant consequences for the folding of the HBD. The dissociation kinetics of the receptor protein dimer are markedly altered by ligand binding, suggesting that one effect of ligand may be to increase the kinetic stability of the complex, and thereby increasing its probability of modulating gene transcription.